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# TEPOXALIN, A NOVEL IMMUNOMODULATORY COMPOUND, SYNERGIZES WITH CSA IN SUPPRESSION OF GRAFT-VERSUS-HOST REACTION AND ALLOGENEIC SKIN GRAFT REJECTION

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**Tepoxalin, a dual 5-lipoxygenase and cyclooxygenase inhibitor with nonsteroidal antiinflammatory effects, has recently been shown to suppress NFκB transactivation and inhibit T cell proliferation via a mechanism very different from cyclosporine (CsA). In this report, we demonstrate that this novel immunosuppressive effect of tepoxalin is manifested in *in vivo* transplantation models. Tepoxalin suppressed murine spleen cell proliferation in a mixed lymphocyte reaction (MLR) with an $IC_{50}$ of 1.3 μM. Coadministration of tepoxalin and CsA in MLR cultures showed an additive inhibitory effect. Oral administration of tepoxalin at 12 mg/kg/day to mice suppressed local graft-versus-host (GVH) responses by about 40% (n=10). Combination of tepoxalin and CsA at suboptimal doses synergized their immunosuppressive effects on GVH responses (n=20). In skin transplantation, the median survival time of allogeneic BALB/cByJ ($H\text{-}2^d$) mouse skin grafted onto C3H/HeJ ($H\text{-}2^k$) mice was 10.5 days (n=8), and was prolonged to 15.0 days (n=9) for recipient mice administered tepoxalin at 50 mg/kg/day. Coadministration of suboptimal doses of tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) prolonged skin graft rejections dramatically (55% of the grafts survived for more than 40 days, n=9). Taken together, these results demonstrate that tepoxalin is a potent immunomodulatory compound that, when combined with CsA, provides synergistic immunosuppressive activity. The fact that tepoxalin and CsA act on different transcription factors, NFκB and NFAT respectively, might explain the synergistic suppressive effects when both compounds were used. Tepoxalin could be an important addition to the cohort of immunosuppressive therapies currently used in solid organ and bone marrow transplantations.**

The immune response in transplantation, which results in graft rejection and graft-versus-host (GVH)* response, is primarily triggered by T cells through recognition of alloantigens (*1–4*). Suppression of immune response could be achieved using agents interfering with T cell activation and effector functions. The use of cyclosporine (CsA) as an immunosuppressant in transplantation has been documented (*5, 6*). CsA inhibits T cell activation by inhibiting the nuclear translocation of the nuclear factor NFAT (*7, 8*). However, CsA has associated toxicities and side effects when used at therapeutic doses (*9*). Compounds that suppress T cell–mediated immune response with mechanisms different from that of CsA will undoubtedly be valuable additions to the cohort of the current regiments.

Tepoxalin (5-[4-chlorophenyl]-N-hydroxy-[4-methoxyphenyl]-N-methyl-1-H-pyrazole-3-propanamide) was discovered originally as a dual inhibitor of 5-lipoxygenase (LO) and cyclooxygenase (CO) and exhibits potent nonsteroidal antiinflammatory activities in animal models of adjuvant arthritis (*10–12*). Recently we found that tepoxalin also inhibits OKT3-induced T cell proliferation via a mechanism very different from that of CsA (*13*). CsA is known to block IL-2 production after activation of T cells through TCR/CD3, whereas tepoxalin inhibits IL-2 induced signal transduction (*13*). An in-depth investigation of the mechanism of action reveals that tepoxalin inhibits predominantly NFκB activation (*14*), whereas CsA is most effective in blocking NFAT transactivation (*7, 8*). Because of these different mechanism of actions, a possible additive/synergistic effect of the combined tepoxalin and CsA treatment is expected. In this report, we demonstrate that tepoxalin is indeed effective in suppressing mixed lymphocyte reactions (MLR), GVH responses, and allogeneic skin graft rejections in mice. The synergistic effect of tepoxalin and CsA in immunosuppression was also studied. The possible mechanism of tepoxalin in immunosuppression and its potential clinical application are discussed.

## MATERIALS AND METHODS

*Mice.* Inbred C57BL/6J, C3H/HeJ, and BALB/cByJ mice and B6D2$F_1$/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Those used in experiments were male mice at about 6–10 weeks of age that weighed 18–25 gm.

*Preparation of test compounds.* Tepoxalin, naproxen, and zileuton were synthesized by the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). CsA (Sandimmune i.v.) was from Sandoz (Quebec, Canada). For MLR experiments, stock solutions of tepoxalin, naproxen, and zileuton were prepared in DMSO at 30 mM and diluted to working concentrations in culture medium at the time of experiments. DMSO at concentrations equivalent to those of the test compounds were used as controls in MLR assays. For experiments of GVH responses and skin graft rejections, micronized tepoxalin and naproxen were suspended in 0.5% methylcellulose (Sigma, St. Louis, MO) at concentrations of 5 mg/ml or lower. The vehicle control was the equivalent volume of 0.5% methylcellulose. Zileuton was dissolved in 50% polyethylene glycol 200 (Sigma, St. Louis, MO), and the corresponding vehicle control was the equivalent volume of polyethylene glycol 200. CsA was diluted in saline. All compounds were dissolved in vehicle just prior to administration to mice at volumes of

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* Abbreviations: CsA, cyclosporine; CO, cyclooxygenase; GVH, graft-versus-host; LO, 5-lipoxygenase; MLR, mixed lymphocyte re-

*Lymphocyte proliferation assays.* Single-cell suspensions from mouse spleens were washed once with PBS and then resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 $\mu$M 2-mercaptoethanol. Responder spleen cells ($2.5\times10^5$) from C57BL/6J mice (H-$2^b$) were stimulated by $2.5\times10^5$ irradiated (2000 rads) spleen cells from B6D2$F_1$/J mice (H-$2^{b/d}$). The responder and the stimulator cells were cocultured in 250 $\mu$l medium containing various concentrations of the tested compounds in the 96-well plates (round bottom wells, Corning Inc., NY). After 5 days of stimulation, $^3$H-thymidine was added to the cultures (0.5 $\mu$Ci per well) for 4 hr. Plates were harvested using a Tomtec Harvester 96, MACH II (Tomtec Inc., Orrange, CT) and samples were counted using a Wallac 1205 Betaplate scintillation counter (Pharmacia, Uppsala, Sweden).

*Cell viability test.* Cell viability was assessed with the MTT assay. Spleen cells from C57BL/6J mice were prepared in RPMI 1640 medium supplemented with 5% fetal bovine serum and 50 $\mu$M 2-mercaptoethanol. Spleen cells ($2\times10^5$/well) were stimulated with immobilized anti-CD3 (Pharmingen) in the presence of tepoxalin or its vehicle DMSO in 96-well culture plates (Corning Inc., NY). The MTT assay was conducted by using the Celltiter 96 kit (Promega Corp.), which is based on the conversion of a tetrazolium salt by viable cells into a detectable blue formazan.

*Graft-versus-host reactions.* The GVH assay was based on the method of Dorsch and Roser (*15*). Spleen cells from C57BL/6J mice were injected subcutaneously into the footpads of B6D2$F_1$/J mice. Each footpad was injected with $8\times10^6$ spleen cells in 50 $\mu$l. Seven days later, the draining popliteal lymph nodes were removed, trimmed of fat and weighed. Mice injected in the footpads with saline were used as negative controls. Lymph nodes of these mice were indistinguishable from those injected with syngeneic spleen cells. Tepoxalin was administered orally and CsA was given subcutaneously to mice daily started one day before footpad injection unless otherwise specified.

*Skin graft transplantation.* C3H/HeJ mice (H-$2^k$) were anesthetized by intraperitoneal injection of 2.5% avertin (0.016 ml/g body weight). A grafting bed (about 0.3 cm×1 cm) on the mouse tail was prepared by peeling off skin carefully to avoid bleeding. Tail skin of similar size was peeled from BALB/cByJ mice (H-$2^d$) and then placed over the graft site in an opposite orientation according to the hair growth direction. The grafted skin was protected by a plastic tubing (diameter 0.5 cm, length 3 cm) held in place by wound clips for 5 days. Skin grafts were examined and scored daily. A graft was scored as being rejected when more than 80% of the graft was necrotic. CsA was given subcutaneously to mice daily starting one day before skin transplantation until rejection of grafts. Tepoxalin was given orally one day before transplantation and then daily starting one day after transplantation until graft rejection.

*Data presentation and statistics.* Data were analyzed using one-tailed Dunnett's tests. A parametric version was used if data were normally distributed as assessed by the Wilk-Shapiro test. Data which did not meet the assumptions of normality were tested using a nonparametric version of the Dunnett's test.

## RESULTS

*Inhibition of MLR proliferations by tepoxalin.* We recently reported that tepoxalin suppresses T cell proliferation and inhibits the activity of the transcription factor NF$\kappa$B (*13, 14*). T cell activation and proliferation are critical for the initiation of an antigen specific immune response. The transcription factor NF$\kappa$B is also known to be involved in regulating the expression of many target genes in an immune response (*16, 17*). The possible immunosuppressive effect of tepoxalin was therefore studied. To determine whether tepoxalin is capable of inhibiting the immune response against alloantigens, tepoxalin at various concentrations was tested in MLR proliferation assays. The assay was set up by stimulating C57BL/6J (H-$2^b$) mouse spleen cells with irradiated B6D2$F_1$/J (H-$2^{b/d}$) mouse spleen cells. As shown in Figure 1, tepoxalin inhibited cell proliferation in a dose-dependent fashion with an $IC_{50}$ of 1.3 $\mu$M. The inhibitory effect was not related to cell toxicity. Tepoxalin at concentrations of 25 $\mu$M or less did not affect the viability of anti-CD3 stimulated mouse spleen cells after 24 hr of treatment (Table 1). Since tepoxalin is a dual CO/LO inhibitor (*10*), the possible link of its suppression of MLR proliferation to its inhibition of CO and/or LO was studied. To address this question, the well-known CO inhibitor naproxen and the LO inhibitor zileuton were tested in parallel at doses 10-fold higher than their $IC_{50}$ for suppression of CO or LO in mice, respectively. Neither of these compounds, nor the combination of both of them, had an inhibitory effect on MLR proliferation (Fig. 1).

To further understand the mechanism of action of tepoxalin, the kinetics of tepoxalin in inhibiting MLR proliferation was compared to that of the known immunosuppressant, CsA. As shown in Table 2, the inhibitory effect was not diminished when tepoxalin was added 24–72 hr after the initiation of MLR. In contrast, CsA was effective only if it was added at the beginning of the cocultures. To determine whether tepoxalin and CsA were synergistic in inhibiting MLR proliferation, the two agents were tested in combination. Tepoxalin at 0.5 $\mu$M, 1 $\mu$M, or 2 $\mu$M was tested in combination with varying concentrations of CsA (Fig. 2). CsA alone inhibited the response in a dose-related manner with an $IC_{50}$ of 22 nM. Tepoxalin alone inhibited proliferation by 26% at 0.5 $\mu$M, by 55% at 1 $\mu$M, and by 87% at 2 $\mu$M. When tepoxalin and CsA were present at suboptimal concentrations, the inhibition was clearly additive. This additive effect was less significant at concentrations of the two drugs that were strongly inhibitory on their own.

*Suppression of GVH responses by tepoxalin.* The immunosuppressive effect of tepoxalin as demonstrated in MLR as-

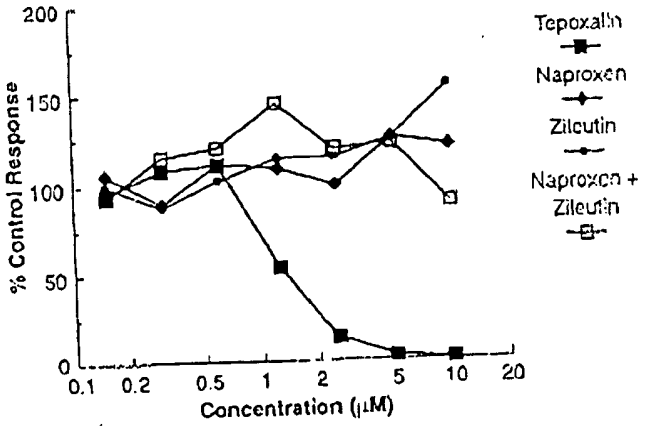


FIGURE 1. Inhibition of MLR proliferations by tepoxalin. Spleen cells from C57BL/6J mice were cocultured in triplicate wells with irradiated spleen cells from B6D2$F_1$/J mice as described in *Materials and Methods*. Varying concentrations of tepoxalin, naproxen, zileuton, or naproxen + zileuton were added to the cultures at the initiation of cultures. $^3$H-thymidine uptake was measured on day 5. Control cultures contained DMSO diluted in a manner similar to that of the compounds. Uptake of $^3$H-thymidine in vehicle controls was about 90,000 cpm. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

TABLE 1. The effective doses of tepoxalin in immunosuppression is not toxic to cells[a]

| Tepoxalin (μM) | Cell viability[b] |
|---|---|
| 100 | 52.0% |
| 50 | 76.4% |
| 25 | 96.5% |
| 12.5 | 103.0% |
| 6.25 | 115.5% |
| 3.12 | 118.4% |
| 1.56 | 136.5% |

[a] Viability of anti-CD3 stimulated C57BL/6J spleen cells treated with tepoxalin for 24 hr was tested in the MTT viability assay.

[b] Cell viability is presented as the percentage of viable cells in tepoxalin treated sample compared with that treated with an equivalent amount of the vehicle, DMSO.

TABLE 2. Inhibitory effect of tepoxalin and CsA on MLR proliferations (% control response)[a]

| | Concentration (μM) | Time of Treatment | | | |
|---|---|---|---|---|---|
| | | 0 hr | 24 hr | 48 hr | 72 hr |
| Tepoxalin | 1.25 | 64.0 | 52.6 | 67.0 | 30.6 |
| | 2.5 | 17.2 | 12.7 | 19.2 | 15.1 |
| | 5.0 | 4.7 | 5.2 | 7.8 | 9.0 |
| Cyclosporine | 0.021 | 40.6 | 65.0 | 97.1 | 133.6 |
| | 0.042 | 18.6 | 99.6 | 93.2 | 148.0 |
| | 0.084 | 5.7 | 61.1 | 84.4 | 119.2 |

[a] Different concentrations of compounds added in MLR cultures at different time points were studied. MLR assays were set up as described in *Materials and Methods*. The MLR proliferations treated with compounds were compared with their vehicle controls. $^3$H-thymidine uptake by proliferating cells in MLR assays was measured. Percentages of control responses are calculated as percentages of (cpm of treated cultures/cpm of vehicle controls).

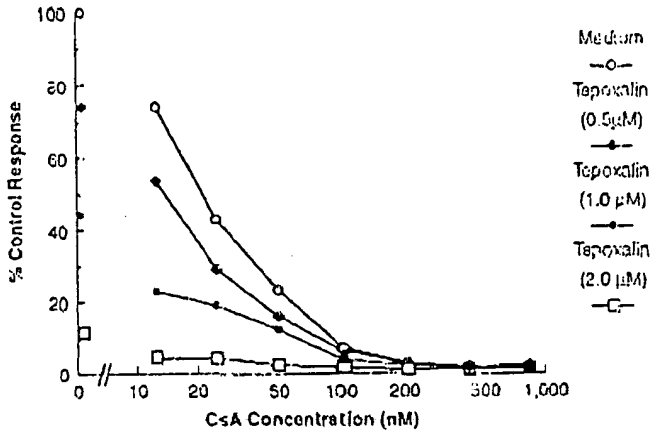


FIGURE 2. Additive inhibitory effects of tepoxalin and CsA in MLR proliferations. Proliferation of C57BL/6J mouse spleen cells after 5 days stimulation with irradiated B6D2F$_1$ spleen cells in medium containing tepoxalin at 0.5 μM, 1 μM, and 2 μM plus varying concentrations of CsA was assayed as described in *Materials and Methods*. The proliferative response in cultures containing no drugs was 80,000 cpm.

says suggests its potential use as an immunosuppressant in clinical therapy. This possible application was verified with *in vivo* murine models of transplantation. A local GVH response was performed by injecting spleen cells from the parental C57BL/6J (H-2$^b$) mice into the footpads of B6D2F1/J (H-2$^{b/d}$) mice. GVH responses were demonstrated by the enlargement of the draining popliteal lymph nodes in recipient mice. The lymph nodes of recipient mice increased significantly by day 2 and continued to increase in size with time. The degree of the local GVH response was measured by weighing the draining popliteal lymph nodes. The lymph nodes of tepoxalin-treated mice did enlarge on day 2 but did not change significantly later on. After 7 days of the local GVH response, lymph nodes from tepoxalin-treated mice were slightly hyperplastic, but were significantly less so than that of the untreated controls (Fig. 3A). GVH responses in mice administered tepoxalin orally at 12–50 mg/kg/day were reduced by about 40% of that in the positive control group. Consistent with the findings in mice, tepoxalin was also effective in rats, with a 30% suppression of this local GVH response at 12 mg/kg/day (data not shown). The immunosuppressive agent CsA administered subcutaneously to mice at 50 and 75 mg/kg/day was shown to suppress GVH response by 42% and 71%, respectively (Fig. 3B). The results suggest that the immunosuppressive effect of tepoxalin at 12 mg/kg/day is comparable to that of CsA at 50 mg/kg/day. To assess whether the inhibitory effect of tepoxalin on GVH responses could be obtained with other CO or LO inhibitors, naproxen and zileuton were again tested in GVH assays. No inhibition was seen with zileuton, naproxen, or a combination of the two compounds (Fig. 3C).

Since tepoxalin appears to act late in MLR assays, the effect of tepoxalin administered early and late in GVH responses was also studied. Similar to the findings in MLR proliferations, tepoxalin given to mice for a minimum of 3 days was sufficient to suppress GVH responses to an extent similar to those treated with tepoxalin throughout the 7-day course of the GVH response (Fig. 4). This short treatment with tepoxalin could be at the early (day −1 to day 1 or 4) or the late (day 4 to day 6) stage of the GVH response. The inhibitory effect of tepoxalin at the late stage of immune responses suggests its mechanism of action to be different from that of CsA. The possible synergism in immunosuppression by tepoxalin and CsA was therefore studied in GVH assays. A much stronger suppression of the GVH response was indeed found in mice treated with both tepoxalin and CsA rather than those treated with either one of the two drugs (Fig. 5). This synergistic effect was particularly significant when a low dose of tepoxalin (6 mg/kg/day) was combined with CsA.

*Prolongation of skin allograft survival by tepoxalin.* The time course of skin allograft rejection in mice is affected by the efficiency of the following two mechanisms: (1) the activation of T cells through recognition of specific alloantigens, and (2) the effector mechanisms mediating tissue distruction. To study the effect of tepoxalin on skin allograft survival, experimental allograft rejection was performed by grafting allogeneic BALB/cByJ (H-2$^d$) mouse tail-skin onto C3H/HeJ (H-2$^k$) recipient mice. For the first 6 days after transplantation, allografts appeared normal and their gross appearance was not different from that of syngeneic grafts. The rejection process became apparent by day 6, with signs of swelling and erythema, and quickly culminated into complete graft necrosis. Different doses of tepoxalin were tested in skin graft rejection assays. As shown in Figure 6, rejection of allografts

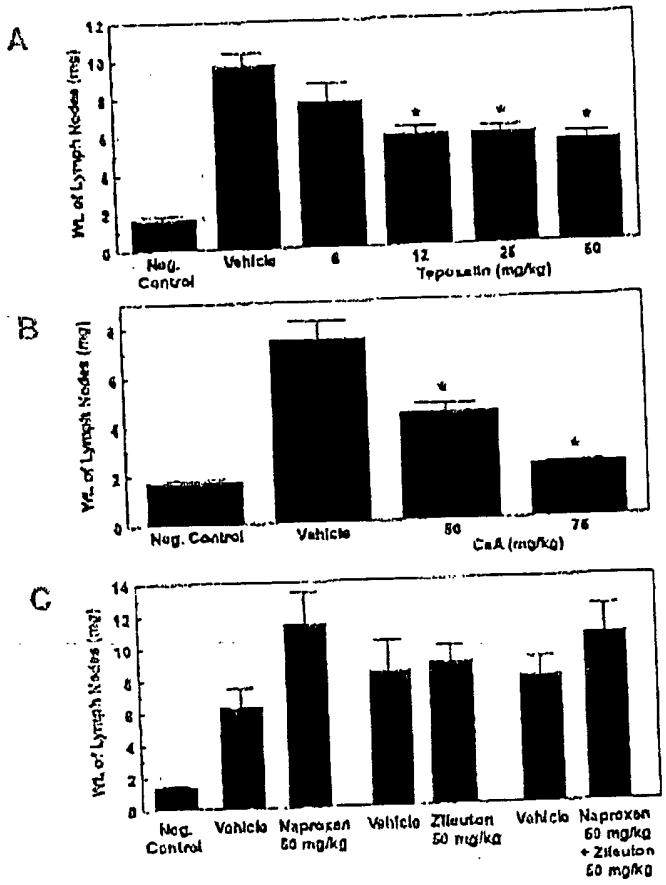


FIGURE 3. Suppression of GVH responses by tepoxalin. A local GVH response was triggered by subcutaneous injection of parental C57BL/6J spleen cells into footpads of $B6D2F_1/J$ mice, and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice injected with saline were used as negative controls. Drugs were given to mice from day −1 to day 6 of the GVH response. (A) GVH responses in mice administered different doses of tepoxalin or vehicle control (0.5% methylcellulose) orally. Ten mice were used per group. The values from mice treated with tepoxalin at 12, 25, and 50 mg/kg/day are significantly different from the vehicle control group (Dunnett's test). Similar results were obtained from more than three repeated experiments. (B) GVH responses in mice given CsA (50 and 75 mg/kg) or vehicle control (saline) subcutaneously. Five mice were used per group. (C) GVH responses in mice given naproxen, zileuton, or the combination of the two drugs at 50 mg/kg/day orally. Mice as vehicle controls for naproxen were treated with equivalent volumes of 0.5% methylcellulose; for zileuton, they were treated with 50% polyethylene glycol 200; and for the combination of drugs, they were treated with both 0.5% methylcellulose and 50% polyethylene glycol 200. Five mice were used per group. The column bars represent the standard errors. Asterisks indicate a $P$ value of $<0.05$.

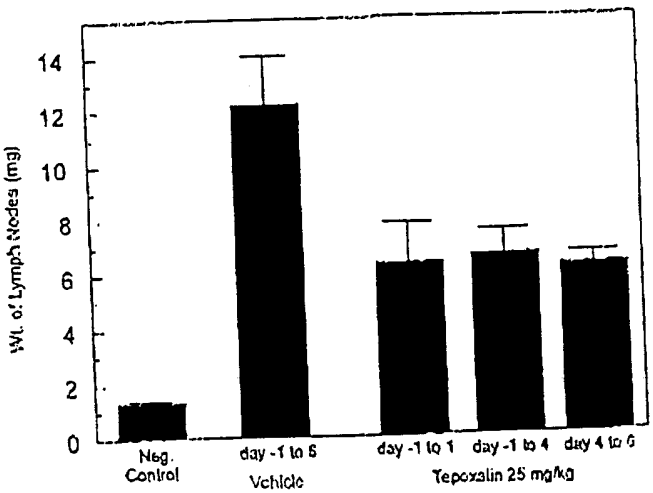


FIGURE 4. Effective suppression of mouse GVH response by short treatments with tepoxalin. The GVH response was induced by injection of C57BL/6J spleen cells into the footpads of $B6D2F_1/J$ mice and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice injected with saline instead of spleen cells were used as negative controls. Tepoxalin (25 mg/kg) was administered orally to mice at different time schedules as shown. GVH responses in mice treated with vehicle (0.5% methylcellulose) were used as positive controls. Five mice were used per group. The column bars represent the standard errors.

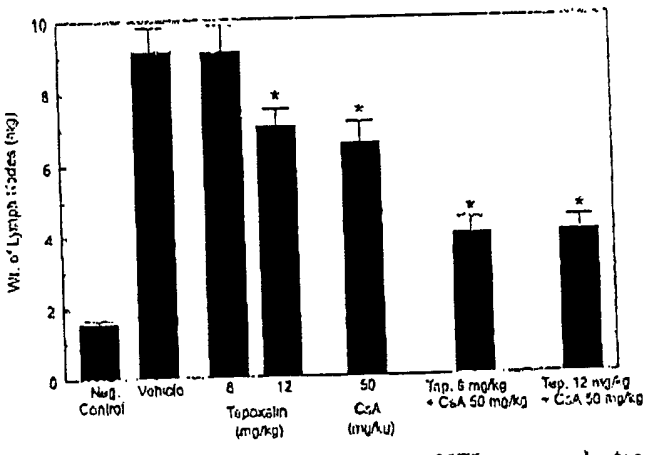


FIGURE 5. Synergistic suppression of mouse GVH responses by tepoxalin and CsA. The GVH response was induced by injection of C57BL/6J spleen cells into the footpads of $B6D2F_1/J$ mice and was measured by weighing the draining lymph nodes as described in *Materials and Methods*. Mice were treated with CsA (50 mg/kg) or tepoxalin (6 or 12 mg/kg) alone, or the combination of tepoxalin (6 or 12 mg/kg) and CsA (50 mg/kg). Mice injected with spleen cells and treated with vehicles were used as positive controls. Mice injected with saline instead of spleen cells were used as negative controls. Twenty mice were used per group. The column bars represent the standard errors. Asterisks indicate a $P$ value of $<0.05$. Similar results were obtained from repeated experiments.

in the placebo-treated group started on day 7. About 50% of the allografts in the placebo group were rejected on day 10. Tepoxalin at doses of 12.5 and 25 mg/kg/day did not have a significant effect in prolonging graft rejection. When tepoxalin at 50 mg/kg/day was administered to mice, a significant prolongation of skin graft rejection was observed. The median survival time of skin grafts, defined as the time point at which 50% of the grafts are rejected, was 10.6 days in the placebo-treated group and was 15.0 days for the group of mice treated with tepoxalin at 50 mg/kg/day ($P<0.05$). Furthermore, a combination of tepoxalin and CsA at low doses showed a dramatic prolongation of allogeneic skin graft rejection (Fig. 7). About 52% of the mice treated daily with

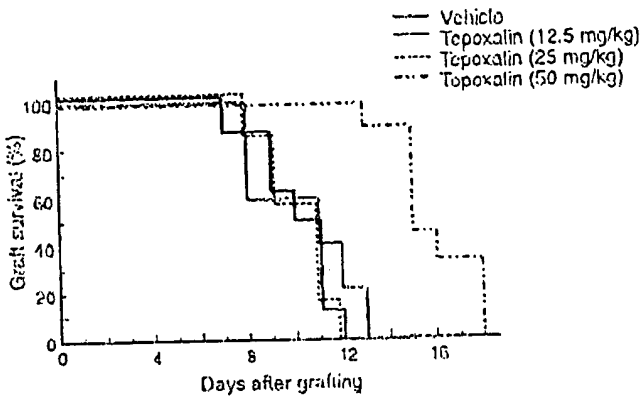


FIGURE 6. Prolongation of skin graft rejections by tepoxalin. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice and rejection of the grafted skin was scored as described in *Materials and Methods*. Different doses of tepoxalin were administered orally to C3H/HeJ recipient mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Mice given the vehicle (0.5% methylcellulose) orally were used as controls. About ten mice were used per group. Data presented were taken from one of the three repeated experiments. Results obtained from all three experiments were similar. Prolongation of skin rejection in mice treated with tepoxalin 50 mg/kg was significant ($P<0.05$, Dunnett's $t$ test).

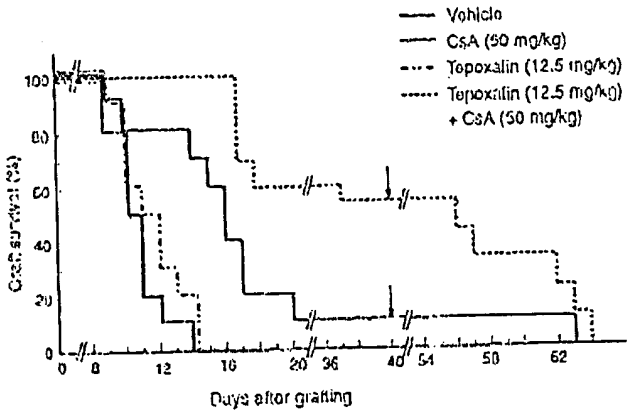


FIGURE 7. Enhanced prolongation of allogeneic skin graft rejection by tepoxalin and CsA. BALB/cByJ mouse tail skin was grafted onto the tail of C3H/HeJ mice as described in *Materials and Methods*. Tepoxalin (12.5 mg/kg) alone, CsA (50 mg/kg) alone, or tepoxalin (12.5 mg/kg) plus CsA (50 mg/kg) were administered to C3H/HeJ mice the day before and after skin transplantation, and then daily until skin grafts were rejected. Tepoxalin was given orally and CsA was given subcutaneously. For recipient mice with skin grafts surviving for more than 40 days, drug administration was discontinued from day 40, as shown by arrows. About ten mice were used per group. Enhanced prolongation of skin rejection was also observed for the combination of tepoxalin (25 mg/kg) and CsA (50 mg/kg) (data not shown).

tepoxalin (12.5 mg/kg/day) and CsA (50 mg/kg/day) retained the allogeneic skin grafts on day 40 after transplantation. To determine whether immunotolerance to skin grafts is generated by the combined drug treatment, drug dosing was discontinued after day 40 of transplantation. Skin graft rejection was noticeable on day 16 and all the grafts were rejected on day 24 after drug cessation (Fig. 7). The results suggest that the combination of tepoxalin and CsA potentiates the immunosuppressive effect, but does not induce immunotolerance to the grafts.

## DISCUSSION

In this report, we demonstrate that tepoxalin is effective in suppressing the immune responses in murine models of GVH reaction and allogeneic skin graft rejection. This immunosuppressive activity is not seen with other inhibitors of CO or LO.

To study the mechanism of immunosuppression by tepoxalin, we used the *in vitro* mixed lymphocyte reaction, which measures the proliferative response of parental strain C57BL/6J spleen cells when stimulated by B6D2F$_1$/J spleen cells. Tepoxalin inhibited the alloantigen-driven proliferative response in a dose-related manner with an $IC_{50}$ of 1.3 $\mu$M and a complete inhibition at 5 $\mu$M. A similar inhibition was seen with CsA, which had an $IC_{50}$ of approximately 22 nM and a complete inhibition at about 200 nM. However, there were differences in the kinetics of the inhibitions seen with the two compounds. Tepoxalin exerted the same degree of inhibition if added any time up to 72 hr after the set-up of MLR cultures, CsA was only inhibitory if added at the initiation of the MLR cultures. IL-2 production by T cells occurs early following activation, reaching peak levels by 24 hr of culture (*18, 19*). CsA has been known for its inhibitory effect on IL-2 production (*7, 20, 21*) and is therefore expected to affect T cells during the first 24 hr of activation. The fact that tepoxalin inhibits proliferation late in MLR assays suggests its inhibition of later events in T cell activation. One possibility is that the IL-2-mediated signal transduction pathway is affected by tepoxalin, which has been shown on human lymphocytes in our previous report (*13*).

GVH disease is a common problem in bone marrow transplantation that leads to frequent morbidity and mortality (*22*). Skin grafts trigger strong immune responses and have been one of the most difficult grafts in transplantation (*3*). The immunosuppressive activity of tepoxalin was demonstrated in murine models of GVH responses and allogeneic skin graft rejections. Tepoxalin was found to inhibit GVH responses at 12 mg/kg/day and to prolong skin graft rejections at 50 mg/kg/day. The possibility that tepoxalin blocks a later event in immune response is again implicated by its suppression of GVH reaction even when it was administered to mice 4 days after the initiation of the response.

Tepoxalin is known to be a dual CO and LO inhibitor with potent antiinflammatory effects (*10*). One of the obvious questions to ask is whether its immunosuppression is due to the inhibition of the CO or LO enzymes. The involvement of CO and LO in the modulation of immune responses remains controversial. Arachidonic acid metabolites produced by these enzymes, such as prostaglandins and leukotrienes, have many biological activities, including the modulation of inflammation and immune response (*23–29*). Indeed several inhibitors of LO have been shown to prolong graft rejection in transplantation (*30–33*). However, it was noticed that those LO inhibitors with immunosuppression activity are also potent antioxidants with inhibitory effects on NF$\kappa$B activity (*34, 35*). Therefore the immunoregulatory effects of these

compounds may not be directly related to inhibition of LO. We compared the effect of tepoxalin with other known LO/CO inhibitors in our studies. Naproxen (CO inhibitor) or zileuton (LO inhibitor), or their combination, did not have any effect on MLR proliferations or GVH responses. We have reported recently that tepoxalin is distinct from other CO and LO inhibitors in its inhibition of NFκB activities (*14*). NFκB is a pleiotropic transactivator of many target genes involved in immune or inflammatory responses (*16, 17*). The immunosuppressive effect of tepoxalin may be attributed to its inhibition of NFκB and not related to the general inhibition of arachidonic acid metabolism.

Taken together, these data show that tepoxalin is an effective immunosuppressive agent. Since the mechanism of tepoxalin appears to be different from CsA in immunosuppression, it suggests a possible combinational use of the two compounds in immunosuppressive therapy. Moreover, tepoxalin is devoid of ulcerogenic actions in gastrointestinal systems that are the common side effects of other NSAID drugs (*11, 12*). The $LD_{50}$ of tepoxalin in mice and rats was more than 400 mg/kg, which is over 10-fold higher than the effective doses used in *in vivo* immunosuppression. Tepoxalin could therefore be an important addition to the existing immunosuppressive therapeutic drugs to enhance the efficacy of treatment and to reduce drug toxicity in transplantation and autoimmunity.

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# COMBINED THERAPY WITH INTERLEUKIN-4 AND INTERLEUKIN-10 INHIBITS AUTOIMMUNE DIABETES RECURRENCE IN SYNGENEIC ISLET-TRANSPLANTED NONOBESE DIABETIC MICE

## ANALYSIS OF CYTOKINE MRNA EXPRESSION IN THE GRAFT[1]

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**Syngeneic pancreatic islet grafts in nonobese diabetic (NOD) mice elicit a cell-mediated autoimmune response that destroys the insulin-producing β cells in the islet graft. IL-4 and IL-10 are cytokines that inhibit cell-mediated immunity. In this study, we evaluated the effects of IL-4 and IL-10 on the survival of syngeneic pancreatic islets transplanted into diabetic NOD mice. Islet grafts survived beyond 18 days and normoglycemia was maintained in 67% (10 of 15) of mice treated with IL-4 plus IL-10, but in none (0 of 20) of vehicle-injected (control) mice. Also, 40% (6 of 15) of the mice treated with IL-4 plus IL-10 were normoglycemic at 30 days after transplantation, compared with 14% (1 of 7) of the mice treated with IL-4 alone, 8% (1 of 13) of the mice treated with IL-10 alone, and none (0 of 20) of the control mice. Histological examination of grafts at 10 days after transplantation revealed periislet accumulations of mononuclear leukocytes and intact islet β cells in grafts from IL-4 plus IL-10-treated mice, whereas islets were infiltrated by leukocytes and the β cell mass was greatly reduced in grafts from control mice. Polymerase chain reaction (PCR) analysis of cytokine mRNA expression in the grafts revealed higher levels of IL-2, IFNγ, and IL-10 mRNA in grafts of diabetic compared with normoglycemic control mice, whereas IFNγ and TNFα mRNA levels were significantly decreased in grafts of IL-4 plus IL-10-treated mice compared with either normoglycemic or diabetic control mice. These results suggest that T helper (Th)1 cells and their cytokine products (IL-2, IFNγ, and TNFα) may promote islet β cell destructive insulitis and autoimmune diabetes recurrence in syngeneic islet-transplanted NOD mice, and that administration of IL-4 plus IL-10 may inhibit diabetes recurrence by suppressing Th1 cytokine production in the islet grafts.**

Insulin-dependent diabetes mellitus (IDDM)* results from destruction of the insulin-producing pancreatic islet β cells by the host's own immune system. Whereas it is not known what may initiate this autoimmune response against islet β cells, there is abundant evidence that IDDM is T cell-dependent (*1, 2*). However, it is unclear which T cells are involved and how they may lead to islet β cell destruction. A variety of immune/inflammatory cells infiltrate the pancreatic islets and constitute the insulitis lesion (*3, 4*). There is evidence in human patients with IDDM (*5–8*) and in animals with spontaneous IDDM resembling the human disease—the nonobese diabetic (NOD) mouse and the biobreeding (BB) rat (*9–22*)—that islet β cell destruction may involve heterogeneous effec-

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* Abbreviations: BB, Biobreeding; CFA, complete Freund's adjuvant; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; PCR, polymerase chain reaction; Th, T helper.